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Preparation of stem cell aggregates with gelatin microspheres to enhance biological functions

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Short / Running Title: Stem cell aggregates containing microspheres

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Abstract

The objective of this study is to improve the viability and osteogenic differentiation of rat bone marrow-derived mesenchymal stem cells (MSC) cultured by use of gelatin hydrogel microspheres. Gelatin was dehydrothermally crosslinked at 140 °C for 48 hr in a water-in-oil emulsion state. When cultured with the gelatin hydrogel microspheres in the round U-bottom well of 96-well multiplates coated poly(vinyl alcohol), MSC formed an aggregate incorporating microspheres homogeneously. The viability of cell aggregate was significantly high compared with that of aggregates formed without microspheres. The MSC proliferation in the aggregates depended on the number and the diameter of microspheres incorporated. Higher MSC proliferation was observed for the aggregates incorporating higher number of larger gelatin microspheres. When evaluated as a measure of aerobic glycolysis, the ratio of L-lactic acid production/ glucose consumption of MSC was significantly lower for MSC cultured with gelatin microspheres than those without microspheres. The MSC production of alkaline phosphatase (ALP) and sulfated glycosaminoglycan (sGAG) was examined to evaluate their potential osteogenic and chondrogenic differentiation. The amount of ALP produced was significantly higher for MSC aggregates cultured with the gelatin microspheres than that of MSC cultured without microspheres. On the other hand, the amount of sGAG produced was significantly lower for MSC aggregates containing the microspheres. It is concluded that the incorporation of gelatin hydrogel microspheres prevents the MSC aggregates from the lack of oxygen, resulting in enhanced vitality of MSC aggregation and the consequent

promotion of cells proliferation and osteogenic differentiation.

Keywords: mesenchymal stem cell, aggregate, gelatin microspheres, proliferation, osteogenic differentiation

1. Introduction

With the recent advent of stem cell biology, various stem cells have been available and basic cell researches have become more and more popular to make clear the molecular mechanism of cell proliferation and differentiation. Most of cells is required to form an aggregate where the cell-cell interaction is physiologically promoted for the enhancement of cell survival and functions.[1] For example, embryonic stem cells are generally aggregated to form an embryoid body and consequently, initiate to differentiate into different cell lineages.[2] The aggregation of liver cells to form the spheroid is necessary to enhance the metabolic activity.[3] Cell aggregates produced the proteins of extracellular matrix more efficiently than single cells in the separate form.[4] Looking at the cell structure of body tissue, such as the liver and bone, the cell aggregate functions as the minimum unit of formation.[5] Taken together, the aggregation can manipulate the cell-cell interaction, resulting in enhanced biological formations of cells. The conventional cell culture has been performed in the 2-dimensional system, which is quite different from the local environment of cells in the living tissue. Therefore, some technologies to culture cells in the 3-dimensional fashion have been performed.[6-9] However, when the size of cell aggregates becomes larger, the cells inside the aggregate tend to die because of the lack of oxygen and nutrients.[10, 11] Such bad conditions often make it practically difficult to perform the in vitro cell culture for a long time period although the long-term culture is required for the basic research of cell differentiation and organization.

Gelatin is one of the biodegradable biomaterials which have been extensively used for the medical, pharmaceutical, and cosmetic applications. The biosafety has been proven through the long-term practical usage.[12] Hydrogels with different shapes could be formulated and their feasibility as the cell culture substrate[13-15] and cell scaffold for tissue regeneration[16-20] or the release carrier of growth factors and drugs[21-28] have been experimentally demonstrated. Gelatin microspheres were effective in enhancing growth factor-induced tissue regeneration.[29-31] Based on the biological availability and the easiness of hydrogel formulation, in this study, gelatin was selected as the hydrogel material.

The objective of this research is to obtain fundamental information whether or not the incorporation of microspheres into cell aggregates can affect the viability and functions of cells present in the aggregates. To this end, hydrogel microspheres were incorporated into the aggregates of rat bone marrow-derived mesenchymal stem cells (MSC) to positively affect the conditions of oxygen and nutrients. The surface of cell culture plate was coated with poly(vinyl alcohol) to prevent the cell adhesion onto the plate. Consequently, cells can be naturally interacted with each other to form the aggregates efficiently. During the cell culture, gelatin microspheres were co-cultured to allow them to incorporate into the cell aggregates for better supply nutrients and oxygen to cells in the aggregates. The viability of MSC aggregates incorporating gelatin microspheres was evaluated and compared with that of cell aggregates formed without microspheres. We examine the osteogenic and chondrogenic differentiation of MSC aggregates.

2. Materials and Methods

2.1. Preparation of gelatin hydrogel microspheres

Gelatin microspheres were prepared by the chemical cross-linking of gelatin in a water-in-oil emulsion state according to the method previously reported.[32] Briefly, aqueous solution (20 ml) of 10 wt% gelatin (the isoelectric point = 5.0, the weight-averaged molecular weight = 100,000, Nitta Gelatin Inc., Osaka, Japan) was preheated at 40 °C, and then added dropwise into 600 ml of olive oil (Wako, Ltd., Osaka, Japan) at 40 °C, followed by stirring at 200 or 400 rpm for 10 min to prepare a water-in-oil emulsion. The emulsion temperature was decreased to 4 °C for the natural gelation of gelatin solution to obtain non-crosslinked microspheres. The resulting microspheres were washed three times with cold acetone by combination with centrifugation (5,000 rpm, 4 °C, 5 min) to exclude residual oil completely. Then, they were fractionated in size by sieves with apertures of 20, 32, and 53 μm (Iida Seisakusyo, Ltd., Osaka, Japan) and air-dried at 4 °C. The non-crosslinked and dried gelatin microspheres (200 mg) were treated in a vacuum oven at 140 °C and 0.1 torr for dehydrothermal crosslinking of gelatin according to the method previously reported.[24] The photographs of gelatin hydrogel microspheres in the water-swollen state were taken on the microscope (CKX41, OLYMPUS, Co., Tokyo, Japan). The size of 100 microspheres per each sample was measured by the Image J of computer program (NIH, Bethesda, USA) while the average diameter was calculated from the data.

2.2. MSC Preparation

MSC were prepared from the bone marrow of 3-week-old, male Fischer F344 rats (Shimizu Laboratory Supply, Kyoto, Japan) according to the conventional procedure.[33] Briefly, both the ends of rat femurs were cut away from the epiphysis, and the bone marrow was flushed out using a syringe (21 gauge needle) with 1 ml of 10mM phosphate-buffered saline solution (PBS, pH 7.4). The cell suspension was placed into two 25 cm² flasks (Iwaki Glass, Ltd., Chiba, Japan) by 4 ml of alpha minimum essential medium (α MEM) (Invitrogen, Co., California, USA) supplemented with 15 vol% fetal calf serum (FCS) (Thermo Inc., Waltham, USA), penicillin (50 U/ml), and streptomycin (50 U/ml) (standard medium) and cultured at 37 °C in a 95% air-5% carbon dioxide atmosphere. The medium was exchanged on day 3 after culture and every 3 days thereafter. When became sub-confluent, 7 to 10 days culture later, the cells were detached by treatment with PBS containing 0.25 wt% trypsin and 0.02 wt% ethylenediaminetetraacetic acid for 5 min at 37 °C. The cells were subcultured at a density of 2×10^4 cells/cm². When proliferated to reach a sub-confluence state, cells were used for the following experiments. This was approved and performed according to guidelines of Institute for Frontier Medical Sciences, Kyoto University.

2.3. Preparation of cell non-adhesion surface

Various types of poly(vinyl alcohol), kindly supplied from Unichika Co., Tokyo, Japan,

(Table 1) were dissolved in PBS (1 wt%). The solution (500 μ l/well) was added in each well of 24-well multiplate and incubated at 37 °C for 15 min. Then, the solution was removed by aspirator and the well was washed with PBS (500 μ l/well) twice. The MSC suspension (4×10^4 cells/ml, 500 μ l/well) was placed into the well plate. After 4 hr incubation, the number of cells present in the medium was determined by the fluorometric DNA assay according to the method reported previously[34]. Briefly, cells were carefully washed with PBS 3 times to collect, and stored at -30 °C until to assay. After thawing, the cells were digested in 30 mM sodium citrate-buffered saline solution (pH 7.4, SCS) containing proteinase K (250 μ g/ml) and dodecyl sulfate (0.2 mg/ml) at 65 °C for 12 hr. After the cell sample solution (100 μ l) was mixed with 100 μ l of SCS containing 1mg/ml Hoechst 33258 dye, the fluorescence intensity of the mixed solution was measured on a fluorescence spectrometer (SPECTRA MAX GEMINI EM, Molecular Devices, Sunnyvale CA) at excitation and emission wavelengths of 355 and 460 nm, respectively. The cell number was obtained by use of the calibration curve which had been prepared with cell suspension at determined numbers. Experiments were performed for 3 wells of each sample unless otherwise mentioned. Then, the number ratio of cells non-adhered to those adhered to the PVA-coated well was calculated.

2.4. Preparation of MSC aggregates with or without gelatin microspheres incorporation

PVA with a polymerization degree of 1,800 and percent saponification of 88 mole%, was

dissolved in PBS (1 wt%). This solution (100 μ l/well) was added into each well of 96-well multiplate with a flat bottom or round bottom (U-bottom) and incubated at 37 °C for 15 min. Then, the solution was removed by aspiration and the well was washed with PBS (100 μ l/well) twice. Gelatin microspheres of 17.6 ± 7.4 , 47.9 ± 22.2 , and 106.8 ± 17.8 μ m in diameter and MSC were suspended in the standard medium separately. The suspension of gelatin microspheres (0, 2×10^3 , 2×10^4 , 2×10^5 , and 2×10^6 , 50 μ l/well) was added into the well coated, and then the MSC suspension (2×10^4 cells/ml, 50 μ l/well) was placed and cultured. Experiments were performed for 3 wells of each sample unless otherwise mentioned.

2.5. Preparation of cell aggregate frozen section

MSC (1×10^4 /well) were cultured with 1×10^4 gelatin microspheres (17.6 ± 7.4 μ m in diameter) to form the cell aggregate. After incubation for 7 days, the aggregate was fixed with 4 % paraformaldehyde at 4 °C for 1 hr and embedded with an Optimal Cutting Temperature (O. C. T.) compound (Sakura Finetek Japan Co., Ltd., Japan) and freezed by liquid nitrogen. The frozen sections were prepared by cryotome (CM3050S, Leica, Germany) and stained with TO-PRO-3 (Molecular Probes, Oregon, USA) together with hematoxylin. The section of 10 μ m thickness was viewed by confocal laser scanning microscopy.

2.6. Measurement of live cells number

The number of live cells in MSC aggregates with or without gelatin microspheres was determined by counting the number of cell nuclei after crystal violet treatment.[35] Briefly, mixed solution of 0.2 M citric acid and 0.2 wt% crystal violet was added (100 μ l/well) to each well of the well plate 2, 4, and 7 days after MSC culture (the number of cells seeded initially per well = 1×10^3 and 1×10^4) with or without 1×10^2 , 1×10^3 , and 1×10^4 gelatin microspheres of 17.6 ± 7.4 , 47.9 ± 22.2 , and 106.8 ± 17.8 μ m in diameter. After the crystal violet staining, the cells were lysed in 0.1 wt% Triton X-100 solution in PBS at 37 °C overnight to extract the nuclei from the cells. After pipetting, the number of nuclei collected was viewed on a microscope (CKX31-11PHP, OLYMPUS, Co., Tokyo, Japan) and counted on a hema-cytometer (OneCell Inc. Japan). The nucleus shape of live cells is generally round, which is different from the irregular shape of dead cells nuclei. The live cells can be distinguished from the dead cells by their nucleus shape. As controls, MSC were cultured on each well of 96-well multiplate with flat bottom at a density of 1×10^3 cells/well. Experiments were performed for 3 wells of each sample unless otherwise mentioned.

2.7. Measurement of metabolic activity

MSC were cultured with gelatin microspheres in the similar way to form the cell aggregation. The number of cells seeded initially per well is 1×10^3 . The number of gelatin microspheres with different diameters of 17.6 ± 7.4 , 47.9 ± 22.2 , and 106.8 ± 17.8 μ m is 1×10^4 .

The amount of glucose consumed by MSC aggregates was determined by measuring the

change in glucose concentration in the culture medium with glutest Neo super (Sanwa kagaku kenkyusyo Co., Ltd., Japan) 2, 4, and 7 days after incubation. Similarly, the amount of L-lactic acid produced by MSC aggregates was determined by E-kit (R-Biopharm AG Co., Ltd., Germany) 2, 4, and 7 days after incubation. On the other hand, the number of live cells was determined by the crystal violet staining described above, while the amount of L-lactic acid produced per the number of live cells was calculated. As a measure to evaluate the metabolic activity of cells, the L-lactic acid / glucose ratio was calculated.[35] Experiments were performed for 3 wells of each sample unless otherwise mentioned.

2.8. Differentiation evaluation of MSC aggregates with or without gelatin microspheres incorporation

The cell culture to form the aggregate was performed similarly. The cell aggregates were cultured for 7 days in the osteogenic differentiation medium of α MEM containing 10 mM β -glycerophosphate, 0.2 mM L-ascorbic acid 2-phosphate (Sigma Ltd., Poole, UK), 10 nM dexamethasone, and 10 vol% fetal bovine serum. The number of gelatin microspheres used is 0, 1×10^3 , and 1×10^4 . As a control, MSC were cultured on each well of 96-well multiplate with flat bottom at a density 1×10^4 cells/well. To evaluate the osteogenic differentiation, the amount of alkaline phosphatase (ALP) produced from MSC was determined by the Lab assay ALP kit (Wako, Ltd., Osaka, Japan) according to the procedure reported previously.[36, 37] Experiments were

performed for 3 wells of each sample unless otherwise mentioned.

The culture medium of chondrogenic differentiation was DMEM containing 1 mM sodium pyruvate (Lonza, Tokyo, Japan), 100 nM dexamethasone, 1 vol% ITS PREMIX (Becton, Dickinson and Company), 0.35 mM L-proline, 0.2 mM L-ascorbic acid 2-phosphate (Sigma Ltd., Poole, UK), and 10 ng/ml transforming growth factor (TGF)- β 1. The time periods of chondrogenic culture were 7, 14, and 21 days. The number of gelatin microspheres used is 0 and 1×10^4 . For chondrogenic differentiation, the amount of sulphated glycosaminoglycans (sGAG) produced from MSC cultured with or without gelatin microspheres was determined by the dimethylmethylene blue dye (DMMB) assay previously reported.[37] The cell culture medium (25 μ l) obtained was mixed with 225 μ l of DMMB solution. Then, the solution absorbance was measured at 525 nm using a VERSAmax multiplate reader (Molecular Devices, Sunnyvale CA). The content of sGAG produced was calculated from the absorbance by a calibration curve prepared by use of known amounts of chondroitin sulfate. Experiments were performed for 3 wells of each sample unless otherwise mentioned.

2.9. Statistical analysis

All the statistical data are expressed as the mean \pm standard error of the mean (SEM). The data was analyzed by the unpaired ANOVA to determine the statistical differences between the two mean values and the significant difference was accepted to be the *P*-value of <0.05 .

3. Results

3.1. Characterization of gelatin hydrogel microspheres

Figure 1 shows the typical microscopic pictures of gelatin hydrogel microspheres. The microspheres were of spherical shape and had a smooth surface. The size of microspheres in the swollen condition was 17.6 ± 7.4 , 47.9 ± 22.2 , and 106.8 ± 17.8 μm .

3.2. Preparation of non adhesion surface by PVA coating

Figure 2 shows the non-adhesion behavior of MSC on culture plates coated with different types of PVA. The cell adhesion was inhibited by coating with PVA with higher degrees of polymerization and lower percentages of saponification.

3.3 Preparation of MSC aggregates with or without gelatin microspheres incorporation

Figure 3 shows the microscopic pictures of MSC 24 hr after incubation with or without gelatin microspheres in flat bottom or U-bottom. MSC aggregates were formed only for the U-bottom culture dish, in remarked contrast to the flat one.

Figure 4 shows the confocal laser scanning microscopic pictures of MSC aggregates 7 days after incubation with gelatin microspheres. MSC and microspheres seemed to be distributed homogeneously in the MSC aggregate. The similar condition was observed for the microspheres of

different sizes (data not shown).

3.4. Cell viability of MSC aggregates with or without gelatin microspheres incorporation

Figure 5 shows the time profiles of live MSC number in cell aggregates after culture in the presence or absence of different numbers of gelatin microspheres with various sizes. The number of live cells in the MSC aggregates with gelatin microspheres increased with time whereas that of MSC aggregates without microspheres did not. As the number of gelatin microspheres became larger, the number of live cells increased. The larger number of live cells was observed for gelatin microspheres with larger sizes. For the microspheres with $106.8 \pm 17.8 \mu\text{m}$ at the cell number of 1×10^4 /well, the number of live cells was larger than that of monolayer culture after 7 days incubation.

3.5. Metabolic functions of MSC aggregates with or without gelatin microspheres incorporation

Figure 6A shows the glucose consumption of MSC aggregates cultured with or without gelatin microspheres. MSC aggregates with gelatin microspheres consumed the larger amount of glucose than those without gelatin microspheres. The consumption glucose for cell aggregate with larger microspheres incorporation was similar to that of monolayer cell culture.

Figure 6B shows the L-lactic acid production of cell aggregates cultured with or without gelatin microspheres. MSC aggregates cultured with gelatin microspheres produced the amount of

L-lactic acid less than those without gelatin microspheres. The L-lactic acid production for the cell aggregates cultured with gelatin microspheres was similar to that of MSC monolayer culture.

Figure 6C shows the time profiles of metabolic activity of MSC aggregates containing gelatin microspheres (1×10^3 /well). The L-lactic acid/ glucose ratio of MSC aggregates cultured with gelatin microspheres was significantly lower than that of microspheres-free aggregates and similar to that of MSC monolayer.

3.6. Osteogenic and chondrogenic of MSC aggregates

Figure 7A shows the ALP production of MSC aggregates cultured with or without different numbers of gelatin microspheres. MSC aggregates with gelatin microspheres produced more ALP than MSC in the monolayer culture. No ALP production was observed for MSC aggregates without microspheres.

Figure 7B shows the sGAG production of MSC aggregates with or without gelatin microspheres. MSC aggregates with gelatin microspheres produced less sGAG than MSC aggregates without gelatin microspheres 21 days after incubation.

4. Discussion

The cell adhesion was inhibited efficiently by PVA coating although the extent depended on the PVA type (**Figure 2**). The reason can be explained in terms of PVA adsorption onto the culture

plate of polystyrene. PVA with lower saponification degrees has more units of vinyl acetate residues than that with higher degrees. It is possible that the higher unit number makes PVA molecules more hydrophobic, resulting in enhanced PVA adsorption onto the plate through the hydrophobic interaction. The hydrophilicity of PVA is enhanced with an increase in the degree of PVA polymerization. This would increase the hydrophilicity of plate surface and consequently decrease the cell adhesion. There have been several research reports on the effect of surface hydrophobicity on the cell adhesion.[38-45] Generally, less cell adhesion was observed on the surface of culture plate with higher hydrophilicity. It is necessary for efficient formation of MSC aggregates to modify the bottom shape of culture plate in addition to the surface hydrophilicity by the PVA coating (**Figure 3**). It is likely that the U-shape bottom allowed cells to put together to the center of well. As the result, the frequency of cell-cell contact would increase, resulting in promoted formation of MSC aggregates. For the flat-bottom plate, although the formation of cell aggregates was observed randomly and heterogeneously, the efficiency was very low. This is due to less frequency of cell-cell contact. Although cells do not adhere onto the surface of PVA-coated substrates, it is likely that less frequency of cell-cell contact on the flat surface of substrate does not result in the efficient formation of cell aggregates.

When MSC were cultured together with gelatin microspheres, the aggregates were formed. However, the aggregate formation depended on the size and amount of microspheres used. When the amount of microspheres was excessive, MSC aggregates were not formed (the number of cells

seeded initially per well = 1×10^5 /well) even 7 days after incubation (data not shown). This can be explained in terms of cell-cell interaction. It is reported that the cell-cell contact is one of the factors contributing to the regulation of cell differentiation.[46] It is highly conceivable that the presence of microspheres at an excessive amount physically suppresses the cell-cell contact and interaction, resulting in suppressed formation of cell aggregates. Considering the mechanism of cell aggregate formation, the natural cell-cell interaction is essential. The interaction to form cell aggregates will be achieved only in the presence of microspheres with an appropriate size range at the optimal amount.

Higher glucose levels and lower L-lactic acid production were observed for MSC aggregates formed in the presence of gelatin microspheres. The ratio of L-lactic acid to glucose is a general measure of aerobic metabolism.[35] The lower the ratio is, the higher the aerobic metabolism is. It is apparent in Figure 6, the ratio of cell aggregates with microspheres was significantly lower than that of aggregates without microspheres. The ratio was similar to that of monolayer culture where oxygen is generally abundant in cell culture. It is highly conceivable that the presence of microspheres in the cell aggregates gives cells the better condition of oxygen, resulting in the lower ratio of L-lactic acid/ glucose, in other words, the achievement of an aerobic condition of cell culture. In addition, the presence of microspheres distributed in the cell aggregate would increase the oxygen permeability inside the aggregate. Taken together, we can say with certainty that the number of live cells proliferated in the cell aggregate containing microspheres became higher.

The ALP activity of cell aggregates as one measure of osteogenic differentiation was enhanced (**Figure 7A**), but oppositely chondrogenic differentiation was suppressed (**Figure 7B**). It is well recognized that MSC are likely to differentiate into osteogenic lineages at the normal oxygen level, whereas they are differentiated into chondrocytes at the lower oxygen level.[47-49] For MSC aggregates containing gelatin microspheres, it is possible that the oxygen in the aggregate is at the normal level. On the contrary, MSC aggregates without gelatin microspheres are in a lower oxygen condition because of restricted oxygen permeation. The homogeneous presence of microspheres in cell aggregates would keep the inner oxygen level normal, resulting in promoted osteogenic differentiation of MSC. On the contrary, it is possible that the cell aggregate without microspheres is in an ischemic condition, which tends to allow cells to differentiate into chondrocytes. The amount of sGAG production depended on the incubation time. The reason of time dependence is not clear at present.

Better osteogenesis of MSC for cell aggregates containing microspheres than those in the monolayer culture can be explained from the viewpoint of cell interaction. In the cell aggregate, cells interact in a 3-dimensional manner of natural cell-cell contact whereas cells monolayer cultured interact in a 2-dimensional one. It is highly conceivable that the 3D interaction of natural system gives cells better conditions for osteogenesis.

Stem cell aggregates incorporating microspheres containing transforming growth factor (TGF) - β 1 have been designed to demonstrate their enhanced chondrogenesis.[34, 50, 51] These

papers experimentally confirm that the cell aggregation and combined TGF- β 1 release were effective in inducing the chondrogenic differentiation of cells in vitro. The 3-dimensional cell-cell interaction in the aggregates would enable cells to communicate to each other in the natural fashion, resulting in enhanced activity of TGF- β 1 for chondrogenesis. In this study, the similar positive effect of microspheres incorporation on the culture condition of cells in the aggregates was observed and the metabolic activity of cells was significantly improved. However, the cell differentiation cannot be compared directly because the microspheres used in this study do not contain any growth factors and the mixing method or ratio of microspheres and cells was different. In future, gelatin microspheres containing growth factor will be used and incorporated into the cell aggregates to evaluate the cell differentiation.

The present study demonstrates that the incorporation of gelatin hydrogel microspheres in the MSC aggregates was effective in enhancing the cell viability of aggregates and their metabolic activity. The microspheres enabled MSC in the aggregates to significantly enhance the glucose consumption and decrease the L-lactic acid/ glucose ratio of aerobic measure, which implies the improvement of biological condition of oxygen and nutrient inside the aggregates. The better metabolic condition may affect the osteogenic and chondrogenic differentiation of MSC in the aggregates. However, the cell differentiation of aggregates with or without microspheres incorporation should be investigated more clearly in terms of histological, biochemical, and immunohistochemical examinations.

5. Conclusions

The objective of this study is to improve the viability and osteogenic differentiation of MSC cultured by use of gelatin hydrogel microspheres. When cultured with the gelatin hydrogel microspheres in the round U-bottom well of 96-well multiplates coated poly(vinyl alcohol), MSC formed an aggregate incorporating microspheres homogeneously. The viability of cell aggregate was significantly high compared with that of aggregates formed without microspheres. Higher MSC proliferation was observed for the aggregates incorporating higher number of larger gelatin microspheres. When evaluated as a measure of aerobic glycolysis, the ratio of L-lactic acid production/ glucose consumption of MSC was significantly lower for MSC cultured with gelatin microspheres than those without microspheres. It is concluded that the incorporation of gelatin hydrogel microspheres prevents the MSC aggregates from the lack of oxygen, resulting in enhanced vitality of MSC aggregation and the consequent promotion of cells proliferation and osteogenic differentiation.

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Figure captions

Figure 1. Microscopic pictures of gelatin hydrogel microspheres dispersed in water. The microspheres were separated with sieves with diameters ranging below 20 μm (A), between 20 and 32 μm (B), and between 32 and 53 μm (C). The scar bar is 100 μm length.

Figure 2. Percentage of cells non-adhered onto culture plates coated with various PVAs: (A) the effect of PVA molecular weight. The degree of saponification is 88 %. (B) the effect of PVA saponification. The degree of polymerization is about 1,700.

Figure 3. Microscopic pictures of MSC 24 hr after incubation with (A, B) or without (C, D) gelatin microspheres with $106.8 \pm 17.8 \mu\text{m}$ in diameter ($1 \times 10^4/\text{well}$) in flat (A, C) or U-bottom of culture dish (B, D). The number of cells seeded initially is $1 \times 10^3/\text{well}$. The bar is 500 μm length.

Figure 4. Microscopic pictures of MSC aggregates 7 days after incubation with gelatin microspheres ($1 \times 10^4/\text{well}$). The diameter of microspheres used is $17.6 \pm 7.4 \mu\text{m}$: (A) a confocal microscopic picture of cell aggregate stained with the TO-PRO-3, (B) a light microscopic picture of the corresponding cell aggregate, and (C) the merged picture. The scale bar is 100 μm length.

Figure 5. The time profiles of live MSC number in cell aggregates after cultured in the absence and presence of different numbers of gelatin microspheres with various sizes ($1 \times 10^3/\text{well}$).

(A) The cells were cultured with 0 (\bigcirc), 1×10^2 (Δ), 1×10^3 (\square), and 1×10^4 (\bullet) of gelatin microspheres. The diameter of microspheres used is $106.8 \pm 17.8 \mu\text{m}$. As a monolayer culture, the cells were cultured in each well of flat bottom 96-well multiplates without gelatin microspheres (∇). \star ,

$p < 0.01$; significant against the number of live MSC in cell aggregates cultured without gelatin microspheres at the corresponding time. #, $p < 0.01$; significant against the number of live MSC in cell aggregates cultured with 1×10^2 of gelatin microspheres at the corresponding time. *, $p < 0.01$; significant against the number of live MSC in cell aggregates cultured with 1×10^3 of gelatin microspheres at the corresponding time. §, $p < 0.01$; significant against the number of live monolayer MSC cultured without gelatin microspheres at the corresponding time.

(B) The cells were cultured with gelatin microspheres of 17.6 ± 7.4 (▲), 47.9 ± 22.2 (■), and 106.8 ± 17.8 μm (●) in diameter or without gelatin microspheres (○). The number of microspheres seeded per well is 1×10^4 . As monolayer culture, the cells were cultured in flat bottom 96-well multiplates without gelatin microspheres (▽). *, $p < 0.01$; significant against the number of live MSC in cell aggregates cultured without gelatin microspheres at the corresponding time. †, $p < 0.01$; significant against the number of live MSC in cell aggregates cultured with gelatin microspheres of 17.6 ± 7.4 μm in diameter at the corresponding time. ‡, $p < 0.01$; significant against the number of live MSC in cell aggregates cultured with gelatin microspheres of 47.9 ± 22.2 μm in diameter at the corresponding time. §, $p < 0.01$; significant against the number of live monolayer MSC cultured without gelatin microspheres at the corresponding time.

Figure 6. The metabolic activity of MSC aggregates cultured with or without gelatin microspheres and MSC cultured as monolayer. The cells were cultured with gelatin microspheres of 17.6 ± 7.4 (▲), 47.9 ± 22.2 (■), and 106.8 ± 17.8 μm (●) in diameter or without gelatin microspheres (○).

The number of microspheres seeded per well is 1×10^4 . As monolayer culture, the cells were cultured in flat bottom 96-well multiplates without gelatin microspheres (∇).

(A) The glucose consumption of MSC aggregates cultured with or without gelatin microspheres and MSC cultured as monolayer. \star , $p < 0.05$; significant against the consumption of glucose in conditioned medium of MSC aggregates cultured without gelatin microspheres at the corresponding time. \dagger , $p < 0.05$; significant against the consumption of glucose in conditioned medium of MSC aggregates cultured with gelatin microspheres of $17.6 \pm 7.4 \mu\text{m}$ in diameter at the corresponding time. \ddagger , $p < 0.05$; significant against the consumption of glucose in conditioned medium of MSC aggregates cultured with gelatin microspheres of $47.9 \pm 22.2 \mu\text{m}$ in diameter at the corresponding time. \S , $p < 0.05$; significant against the consumption of glucose in conditioned medium of monolayer MSC cultured without gelatin microspheres at the corresponding time.

(B) The L-lactic acid production of live MSC in cell aggregates cultured with or without gelatin microspheres and MSC cultured as monolayer. \star , $p < 0.05$; significant against the production of L-lactic acid in conditioned medium per the number of live MSC in cell aggregates cultured without gelatin microspheres at the corresponding time.

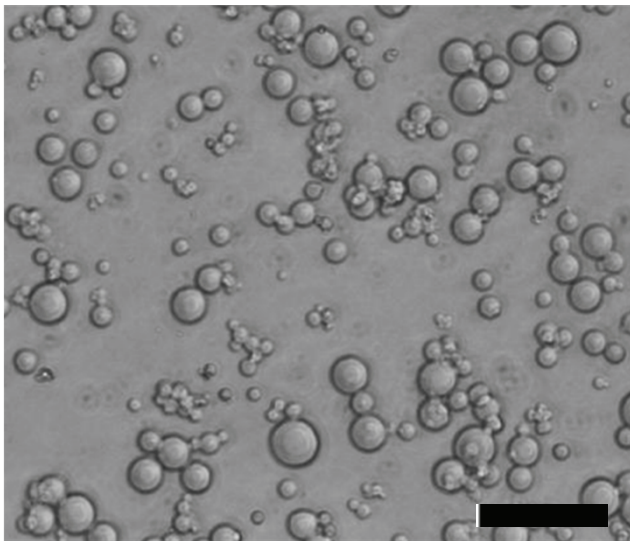
(C) The time profiles of metabolic activity of MSC aggregates containing gelatin microspheres ($1 \times 10^3/\text{well}$). \star , $p < 0.05$; significant against the L-lactic acid / glucose ratio of cell aggregates cultured without gelatin microspheres at the corresponding time.

Figure 7. The differentiation of MSC aggregates with or without gelatin microspheres.

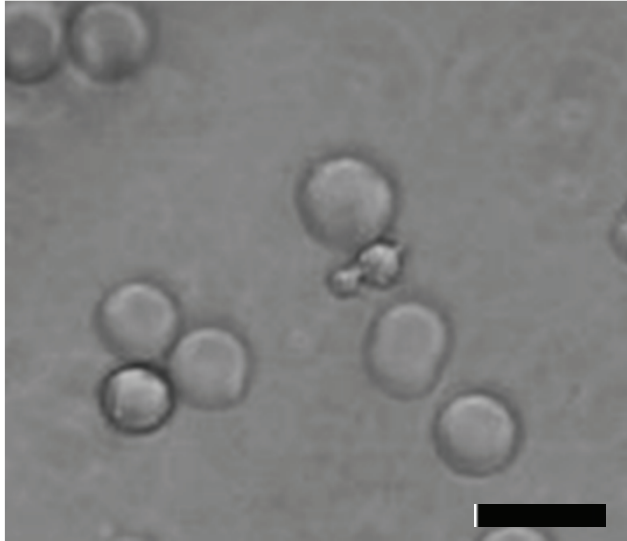
(A) The amount of ALP produced 7 days after incubation of MSC aggregates with or without gelatin microspheres of $106.8 \pm 17.8 \mu\text{m}$ in diameter in the osteogenic differentiation medium ($1 \times 10^4/\text{well}$), and monolayer MSC without gelatin microspheres. The number of cells seeded initially per well = 1×10^4 . *, $p < 0.05$; significant against the amount of ALP produced from MSC aggregates cultured without gelatin microspheres at the corresponding time.

(B) The amount of sGAG produced 7, 14, and 21 days after incubation of MSC aggregates with (●) or without (○) gelatin microspheres of $106.8 \pm 17.8 \mu\text{m}$ in diameter in the chondrogenic differentiation medium ($1 \times 10^4/\text{well}$). The number of cells seeded initially per well = 1×10^4 . *, $p < 0.05$; significant against the amount of sGAG produced from MSC aggregates cultured without gelatin microspheres at the corresponding time.

(A)



(B)



(C)

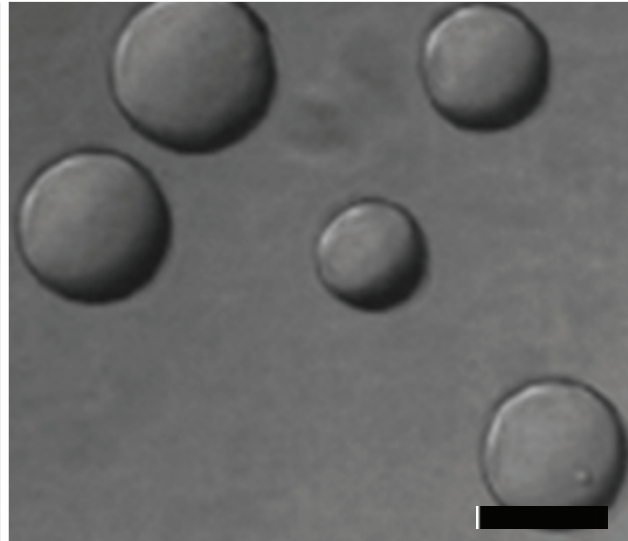


Fig. 1

Table 1. Physicochemical properties of PVA coated

Degree of polymerization	Percentage of saponification (mole%)
500	88
1000	88
1700	96.5
1800	88
1800	84.5

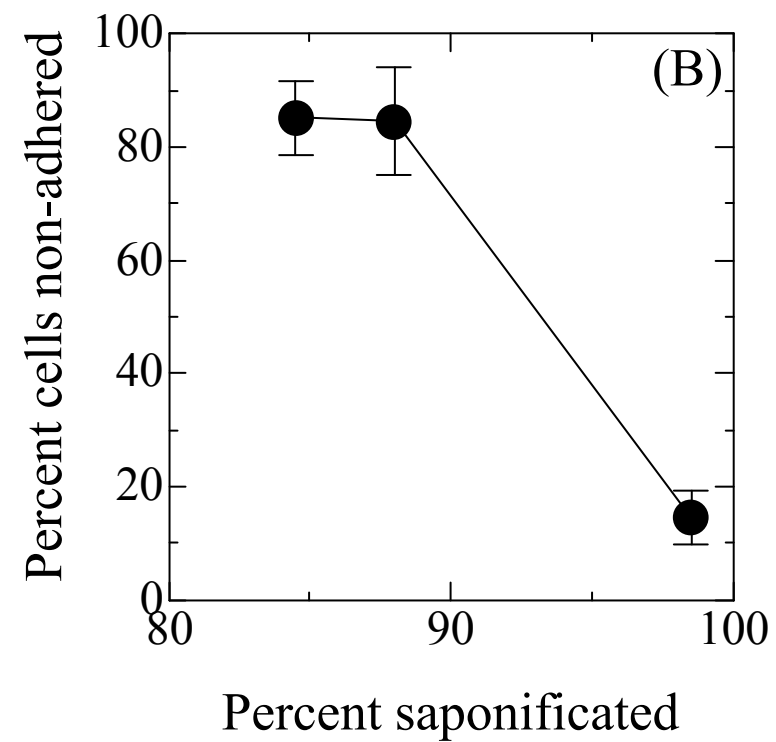
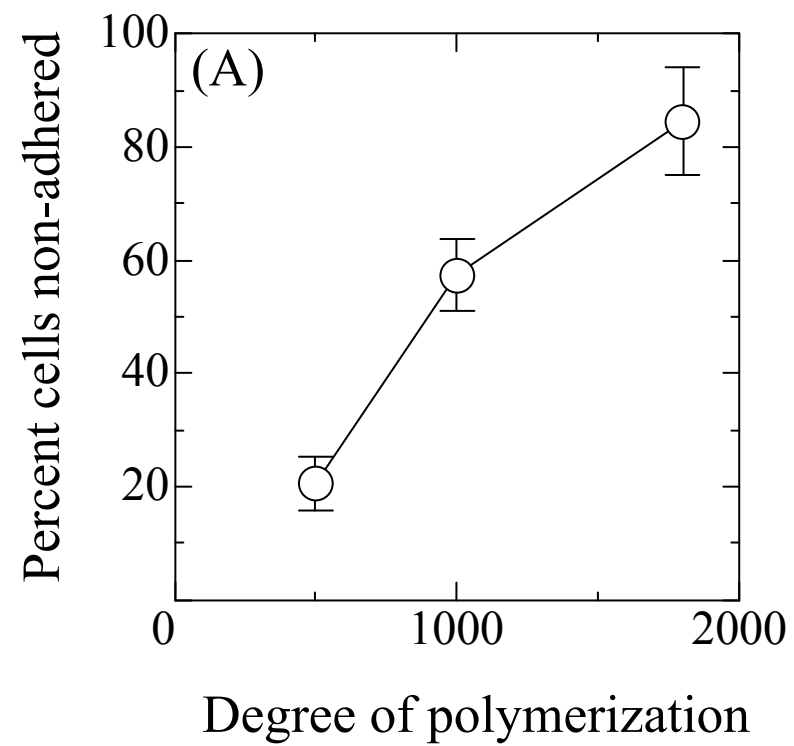


Fig. 2

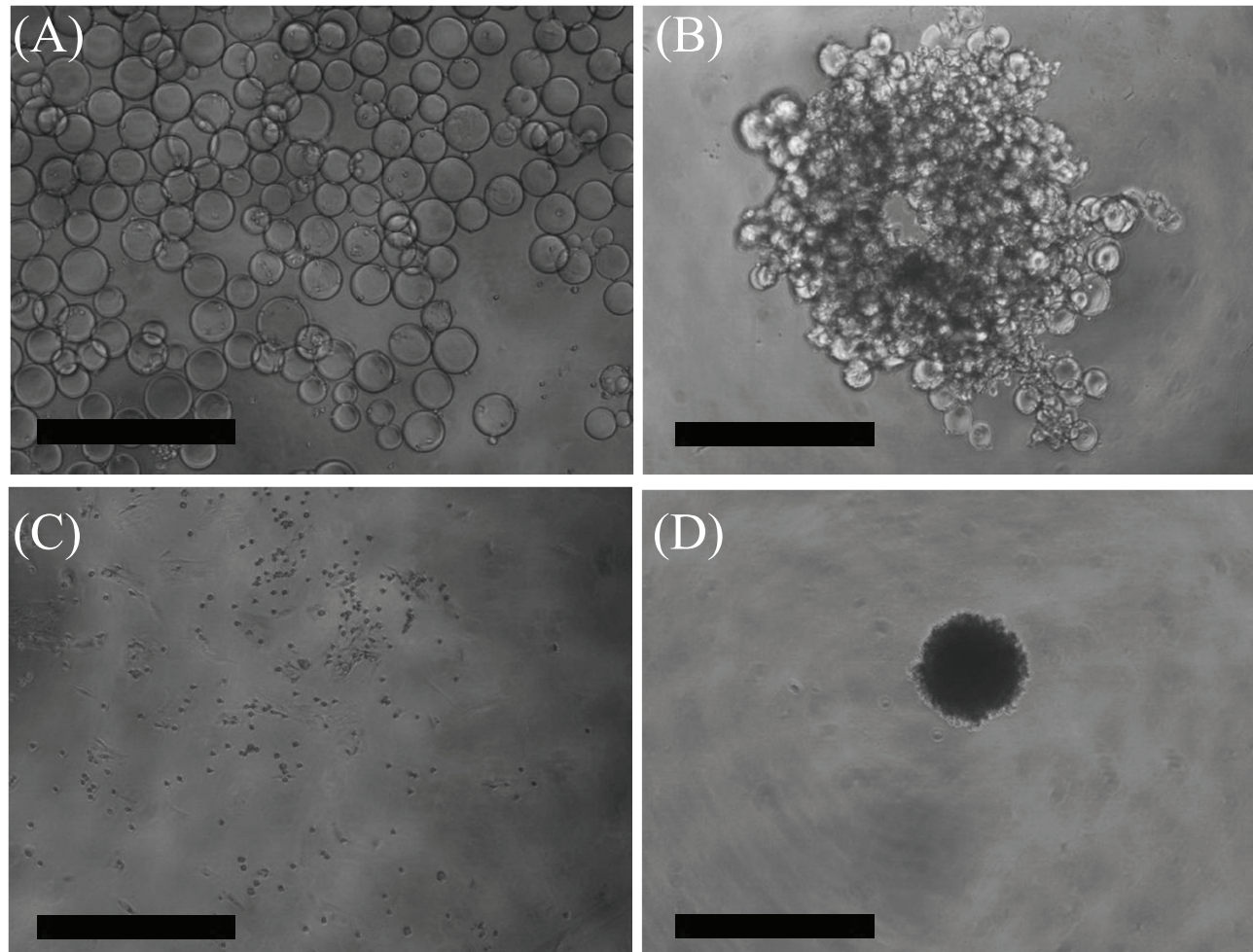


Fig. 3

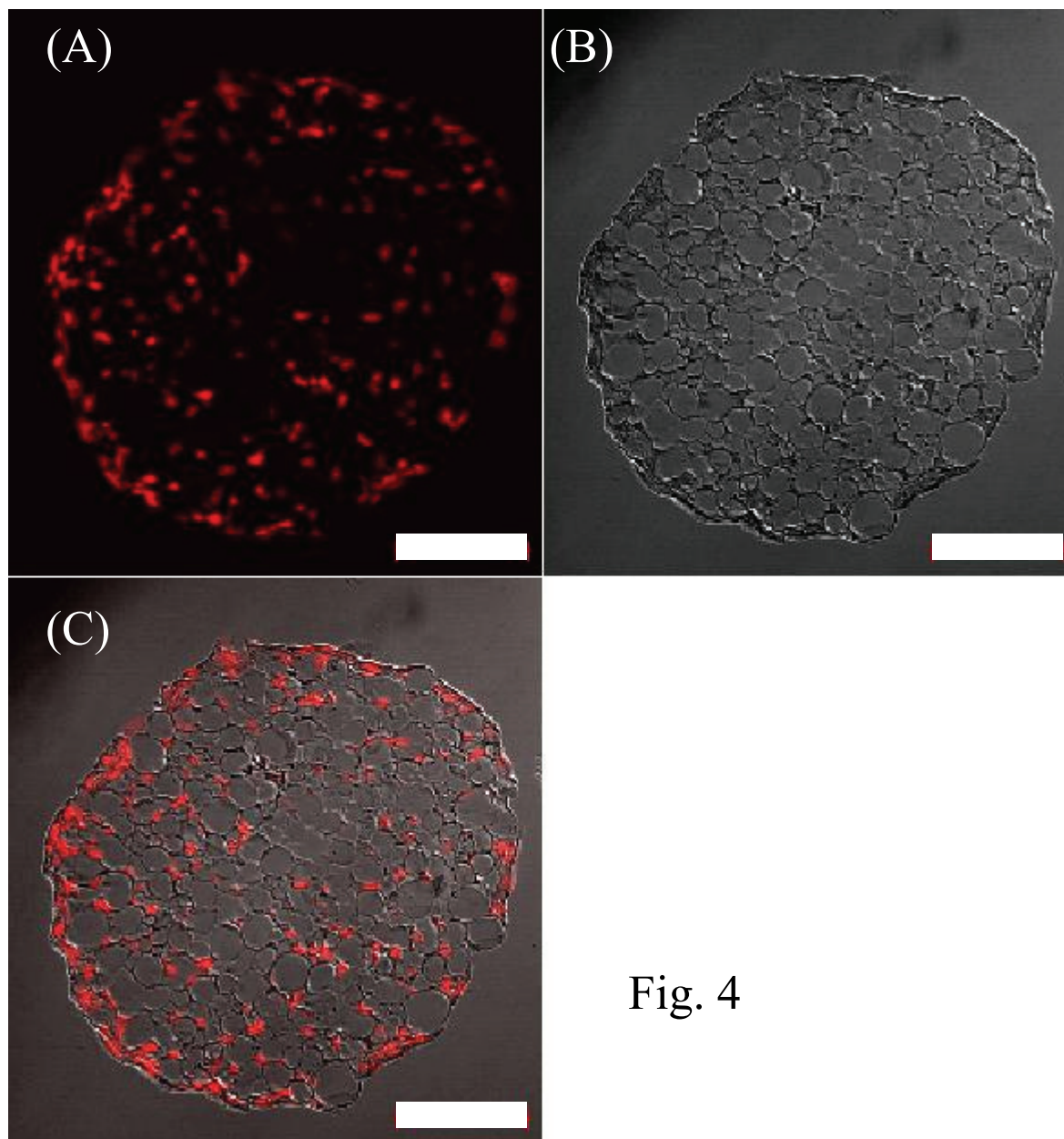


Fig. 4

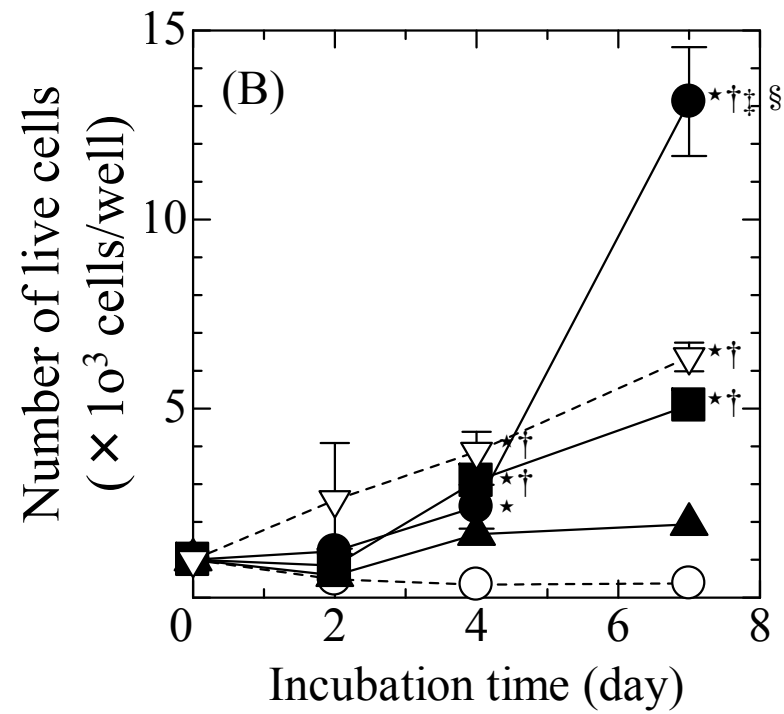
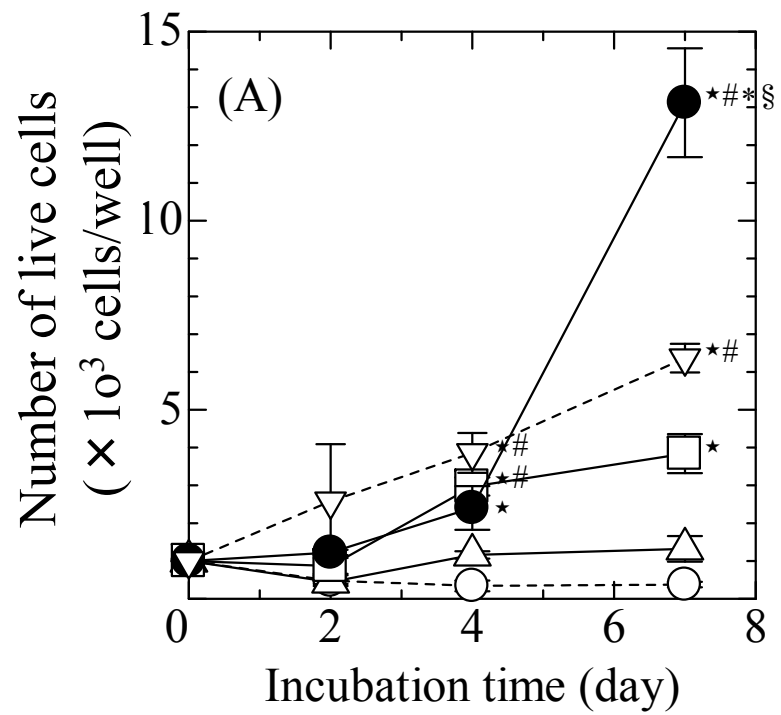


Fig. 5

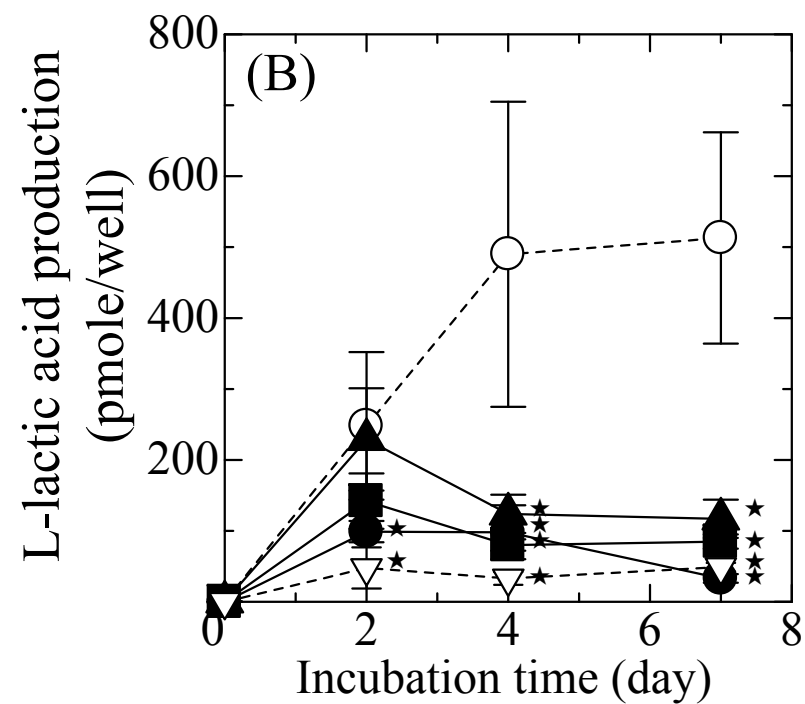
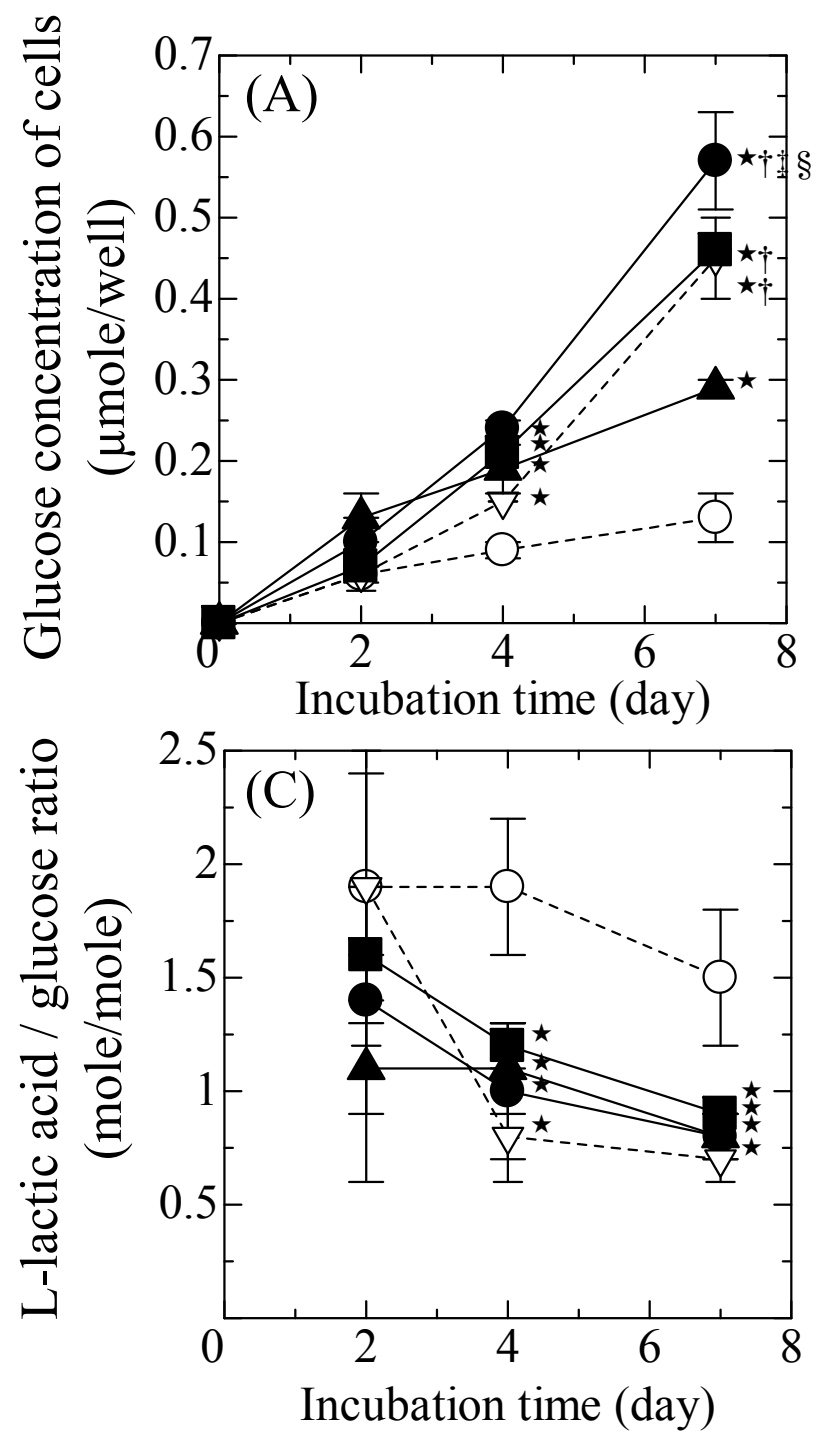


Fig. 6

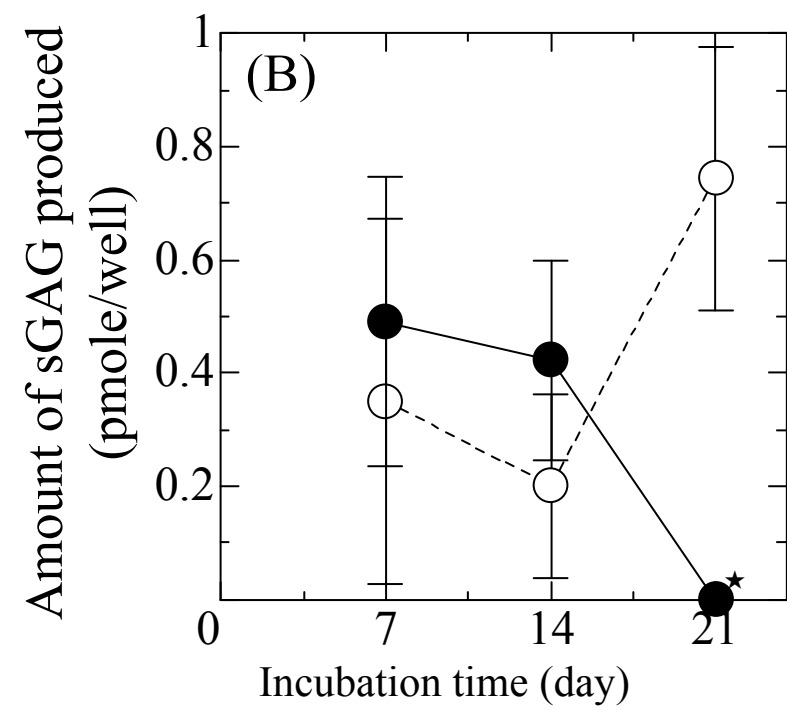
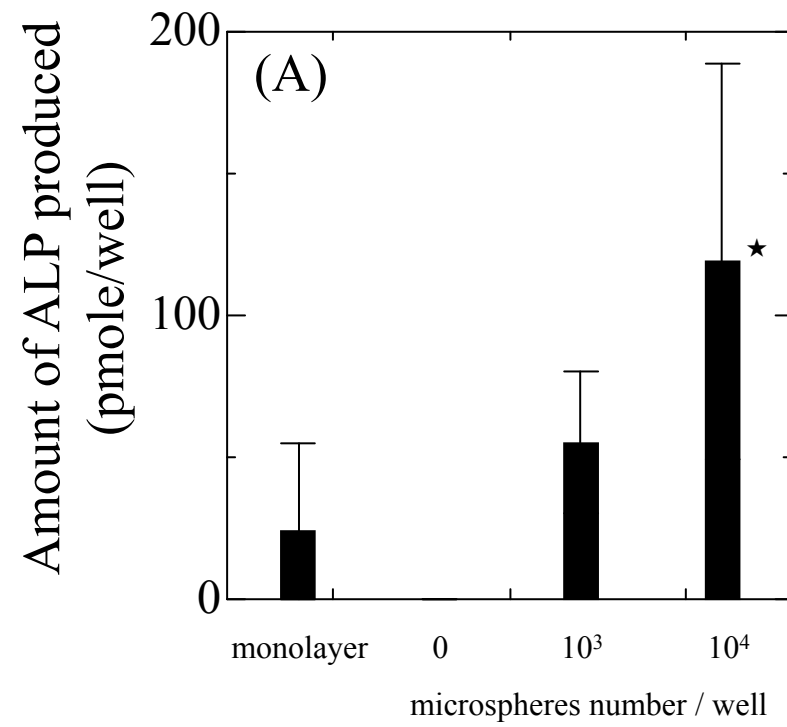


Fig. 7